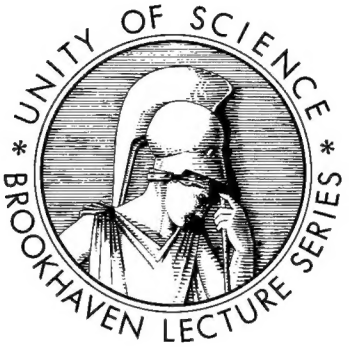
BROOKHAVEN LECTURE SERIES

The Problem of Development

ERNST CASPARI



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FOREWORD

The Brookhaven Lectures, held by and for the Brookhaven staff, are meant to provide an intellectual meeting ground for all scientists of the Laboratory. In this role they serve a double purpose: they are to acquaint the listeners with new developments and ideas not only in their own field, but also in other important fields of science, and to give them a heightened awareness of the aims and potentialities of Brookhaven National Laboratory.

Before describing some recent research or the novel design and possible uses of a machine or apparatus, the lecturers attempt to familiarize the audience with the background of the topic to be treated and to define unfamiliar terms as far as possible.

Of course we are fully conscious of the numerous hurdles and pitfalls which necessarily beset such a venture. In particular, the difference in outlook and method between physical and biological sciences presents formidable difficulties. However, if we wish to be aware of progress in other fields of science, we have to consider each obstacle as a challenge which can be met.

The lectures are found to yield some incidental rewards which heighten their spell: In order to organize his talk the lecturer has to look at his work with a new, wider perspective, which provides a satisfying contrast to the often very specialized point of view from which he usually approaches his theoretical or experimental research. Conversely, during the discussion period after his talk, he may derive valuable stimulation from searching questions or technical advice received from listeners with different scientific backgrounds. The audience, on the other hand, has an opportunity to see a colleague who may have long been a friend or acquaintance in a new and interesting light.

The lectures are being organized by a committee which consists of representatives of all departments of the Laboratory. A list of the lectures that have been given and of those which are now scheduled appears on the back of this report.

Gertrude Scharff-Goldhaber

The drawing on the cover is taken from a 5th Century B.C. relief on the Acropolis in Athens, the "Dreaming Athena," by an unknown sculptor.

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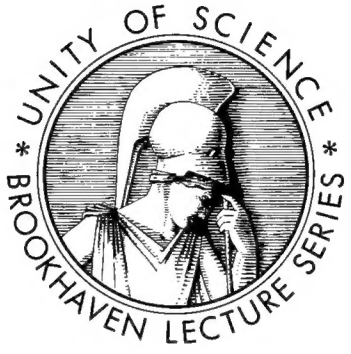

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INTRODUCTION

Just over a dozen years ago several highly significant papers appeared, written by two, at that time, little known scientists. The ideas presented in these papers revolutionized our concepts regarding the chemistry of genetic material by pointing out how the basis of inheritance resides in the ability of certain relatively simple organic molecules to direct their own reproduction. This breakthrough generated a tremendous surge of activity and contributed to the merger of borderline areas of biology, biochemistry, and biophysics into a new branch of science known as molecular biology.

There had been two prior breakthroughs in the young science of genetics. The first was the rediscovery of Mendel's work which resulted in the introduction into biology of new concepts and approaches. Mendel's work contributed to the evidence that factors in the germ cells, subsequently called genes, are responsible for the transmission of each hereditary character, and that in the reproductive process these genes remain unaltered. The second came when Morgan and his collaborators obtained evidence that the genes are located in a linear order in structures called chromosomes. Their work showed that the cell is a unit of heredity and that, generally, every cell of a living organism contains a full complement of genes.

At present geneticists are eagerly looking for another break which would open out vistas into the intricate processes that govern the development of a highly complex organism from the morphologically relatively simple fertilized egg. Geneticists are confident that cellular differentiation is governed by the genetic system of the cell, but our understanding of how that is accomplished is still rudimentary. Our speaker tonight is carrying on research on the processes of development, and he will relate some of his results.

Professor Ernst Caspari was born and educated in Germany. He studied at the Universities of Freiburg, Berlin, Frankfort, and Göttingen, where he received the Ph.D. in 1933. In 1935 he moved to the Medical School of the University of Istanbul where he remained three years. Dr. Caspari came to this country in 1938 and has held positions at Lafayette College, Wesleyan University, the Carnegie Institution Department of Genetics at Cold Spring Harbor, and the University of Rochester. During the war he worked on the induction of mutations for the Manhattan Project. At present Dr. Caspari is Chairman of the Department of Biology at the University of Rochester. He is also a representative of that University on the Board of Trustees of Associated Universities.

(The foregoing introduction was given by Dr. M. Demerec prior to his untimely decease in 1966. It was transcribed from a recording of his remarks and edited by the Biology Department.)

The Problem of Development*

The great progress that has taken place in biology during the past ten years has given us a deeper understanding of the activities of cells in chemical terms. This period of progress was initiated by the introduction of bacteria and viruses as materials for genetic studies. Bacteria and viruses have proved to be particularly convenient organisms for the study of gene action, and most of our present knowledge of the interaction of DNA, RNA, and proteins has resulted from work with microorganisms. The question to which I want to address myself here is not the general one of how far the knowledge obtained in this way can be applied to higher organisms. It is rather the more specific problem of how we can account for the regulated changes in cellular activity and structure which take place in the development of a multicellular animal or plant, in the light of this knowledge.

Experimental embryology is one of the oldest fields of experimental biology and was initiated by Wilhelm Roux in 1883. After rapid progress, particularly in the first quarter of this century, this science became stagnant, since the methods used in classical embryology, defect experiments, explantation, and transplantation, could give information on the interaction of cells and cell groups, but not on the state and activity of the genetic material inside the cells. In the following description, an attempt will be made to view a developmental process from the latter point of view.

A multicellular organism arises from a single cell, the fertilized egg cell. The mechanism whereby an egg cell develops into a multicellular organism is mitotic cell division. For more than fifty years it has been generally assumed that the cell divisions occurring in development are genetically equal, so that all the cells of an organism have the same genetic constitution as the original egg cell. Nevertheless, in the course of development, they become very different from the point of view of shape, structure, chemical composition, and activity. In our own bodies, for example, the cells of the skin are very different from the blood cells and the nerve cells. The question of the origin of these differences between cells presumed to be genetically identical is known as the problem of differentiation. Since we know that different cells have different chemical constitutions, including differences in their protein constitutions and their enzymatic equipment, and since we furthermore know that the formation of specific proteins is an activity of the genes, we must conclude that the problem of differentiation is fundamentally a problem of gene activity. In simplest terms, we may assume that even though all the genes are present in every cell of a multicellular organism, the process of differentiation consists in the activation and repression of different components of the genic complement.

Mechanisms for the activation and repression of particular genes and gene products are known in microorganisms. They form the basis for the regulatory phenomena known as enzyme induction, feedback inhibition, and coordinate repression. These phenomena are being actively investigated at the genetic and molecular level and have been used by Jacob and Monod[1] to construct models for the process of differentiation.

Before discussing the question of how far we can analyze developmental processes at the present time, it may be worth while to mention some of the additional features of development that complicate the analysis. We will have to account for more than differentiation, i.e., the fact that cells of identical genotype show different chemical and morphological properties. In the history of the organism these characters appear in a definite, closely regulated pattern in space and time. It must be assumed that these progressive changes in cells and cell groups appearing at different times are themselves under the control of genes. It is therefore not sufficient to investigate the question of the genetic meaning of differences between cells; the control of timing of the behavior of cells by the genes constitutes a separate problem which is superimposed on and closely interrelated with the first problem.

EYE COLOR IN INSECTS

In the investigation of gene action in multicellular organisms, genes affecting pigments have al-

*The recent work from the author's laboratory described in this paper was carried out under Contract AT(30-1)-2902 with the U.S. Atomic Energy Commission.

ways held an important position. The reason is simply that pigments are chemicals that are visible to the human eye, and, since in all organisms investigated mutants affecting pigments are relatively frequent, the possibility of defining gene action in chemical terms seemed to be less remote in pigment genes than in genes affecting more complex characters. The pigments of insects have an advantage over vertebrate pigments insofar as the latter are contained in migratory cells, while the former are found in cells that remain in fixed positions in the body. Genes affecting the eye pigments have been found in every insect investigated, the greatest number being known in *Drosophila*. I want to deal more specifically with the eye pigments of the meal moth *Ephestia* with which most of the work in our laboratory has been concerned.

The eye pigments of insects consist of two different types of compounds: ommochromes and pteridines. If the eye pigments of adult *Ephestia* are subjected to paper chromatography, three components can be distinguished: a fast migrating yellow spot, a somewhat slower moving red spot, and a slowly migrating purple spot. The yellow spot has been identified as xanthommatin, the slowly migrating one as ommin. The red spot turns out to be composite, consisting of an ommatin of unknown constitution and a pteridine.

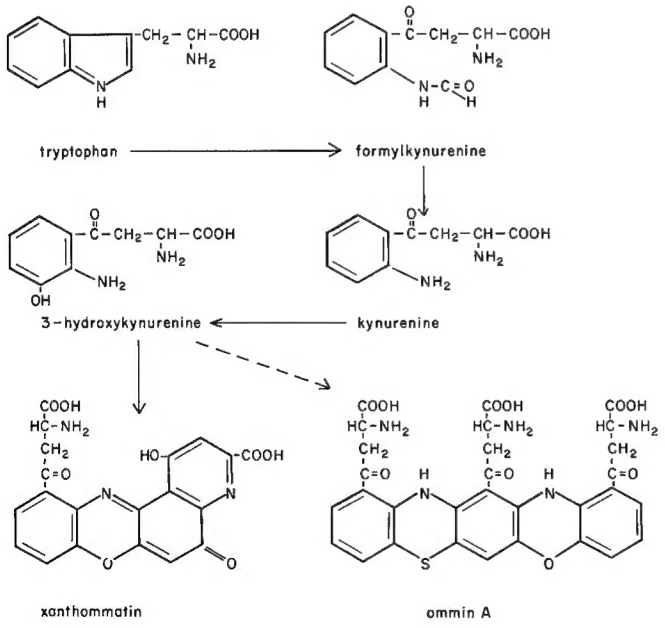

Figure 1. Steps in the biosynthesis of ommochrome pigments.

The biosynthesis of the ommatins and ommins has been well investigated, particularly by the use of genetic mutants. They are derived from tryptophan via kynurenine and 3-hydroxykynurenine (Figure 1). The structures of xanthommatin and ommin, as determined by Butenandt and collaborators,[2] indicate that they are derived from 3-hydroxykynurenine by polymerization of two and three molecules respectively; in the ommins sulfur is introduced into the molecule. The enzyme responsible for the oxidation of tryptophan to formylkynurenine has been demonstrated *in vitro* in *Drosophila* and *Ephestia*,[3–5] the enzyme catalyzing the step formylkynurenine → kynurenine only in *Drosophila*.[6] The enzyme catalyzing the oxidation kynurenine → 3-hydroxykynurenine has been demonstrated only by indirect methods *in vivo*.[7] The condensation of 3-hydroxykynurenine to ommochromes is presumed to occur on granules in the cell; it has been claimed that the reaction occurs *in vitro* in the presence of mammalian mitochondria.

In *Ephestia*, ommochrome pigments appear in different organs at different times. They appear first in the ommatidia (eyes) and hypodermis of the embryo, and can be seen there through the egg shell in the last quarter of the period of embryonic development. The freshly hatched larva contains ommochrome pigments in its ommatidia and in its skin. After the last larval molt, ommochromes appear in an additional organ, the testis sheath, and the pigmentation of the testis increases up to the early pupal stage. The adult eyes, finally, become pigmented in the pupa, the process proceeding gradually until full pigmentation is reached on the eighth day after pupation (Figure 2).

The question of the development of the ommochrome pigments can now be asked in a more precise way: What changes does the chemical constitution of a particular type of cell undergo at a particular time of development, so that it results in the formation of ommochromes in these cells but not in others?

THE PATTERN OF PIGMENT AND ENZYME DEVELOPMENT

It may be assumed, as a simple hypothesis, that the developmental pattern of ommochrome synthesis reflects a developmental pattern of enzyme synthesis. For the analysis of the pattern of enzyme development, it is advantageous that mutant genes

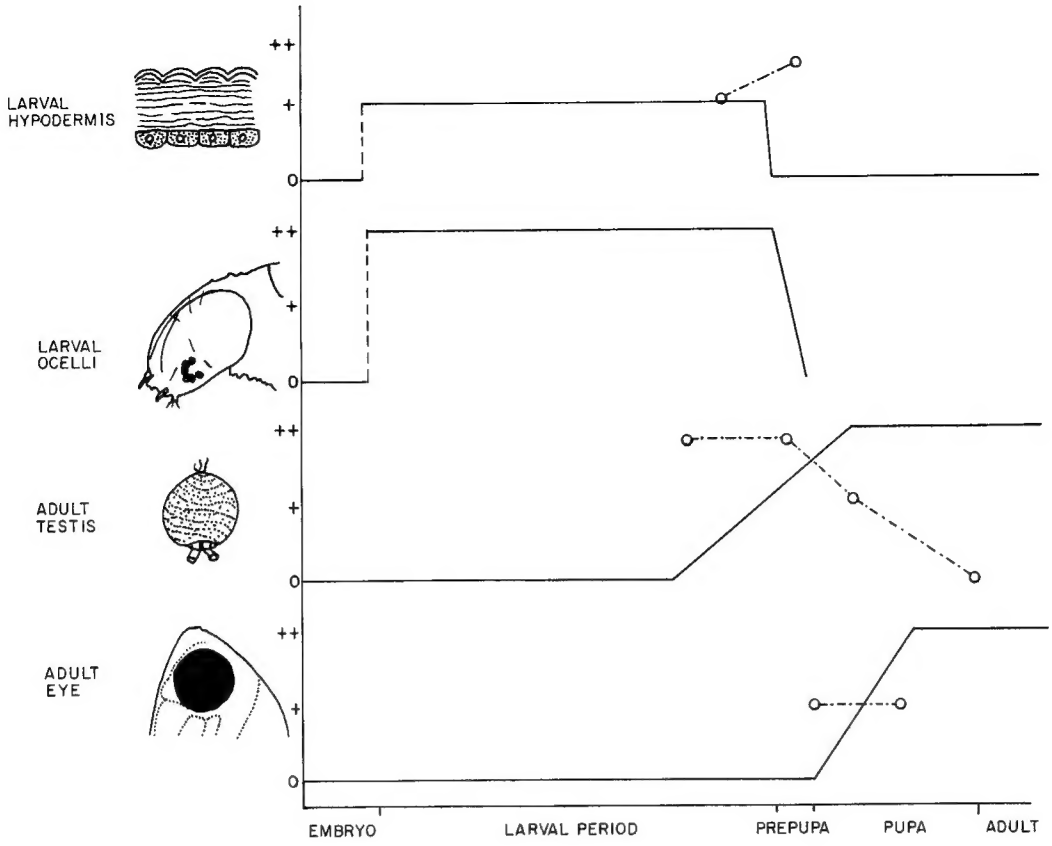

Figure 2. Levels of ommochromes and of tryptophan pyrrolase activity in four organs at different times of development. Abscissa: time, divided according to developmental stages; ordinate: ommochrome content and tryptophan pyrrolase activity, expressed in 3 levels (0=no activity, + = moderate activity, + + = strong activity); ———, content of ommochrome pigment; o–•–•–•–o, tryptophan pyrrolase activity (after Egelhaaf[7]).

blocking particular steps in the synthesis of ommochromes are available, since it is well established that genes control the amino acid sequence in enzyme proteins. The mutant genes *a* in *Ephestia* and *v* in *Drosophila* affect tryptophan pyrrolase, the enzyme responsible for the step tryptophan→formylkynurenine.[3–5] In *Drosophila*, no genetic variation has been found for the enzyme catalyzing the reaction formylkynurenine → kynurenine.[6] No mutant is known in *Ephestia* which blocks the step kynurenine → 3-hydroxykynurenine, but the gene *cn* in *Drosophila* inhibits this step, and analogous mutants are known in the silkworm and in the wasp *Habrobracon*. Finally, the mutant *br* in *Ephestia* inhibits the formation of ommin in the eye without affecting the synthesis of the ommatins, while the allele *rt* inhibits the formation of ommin in the testis but not in the eye.[8,9]

In both *Ephestia* and *Drosophila*, kynurenine is released into the blood, while 3-hydroxykynurenine seems to be released in *Drosophila* but not in *Ephestia*.[7,10] These facts make it possible to test different organs for kynurenine production by implanting them into *aa* or *vv* hosts. These transplanted organs survive and may release kynurenine into the host's blood. The host can use this kynurenine for pigment formation in its own eyes, the amount of pigment formed being proportionate to the amount of kynurenine released. In *Ephestia* larvae, it turns out that the testis is the best source of the substance, but all other organs release the substance to a larger or smaller degree. This is not, however, a very striking finding, since kynurenine may be stored in some organs, so that the release of kynurenine is not necessarily an indication of its production.

Egelhaaf[7] has therefore measured the tryptophan pyrrolase activity of different organs *in vitro* (Figure 2 and Table 1). In the larva, the highest enzyme activity besides that in the testis is found in the fat body and in the spinning glands, both organs that do not form ommochrome pigments. On the other hand it is low in the hypodermis, which

Table 1

Tryptophan Pyrrolase Activity in Different Organs of *Ephestia* at Different Stages of Development (after Egelhaaf[7])

	Last instar larva	Prepupa	Pupa	Adult
Hypodermis	+	++		
Testis	++		+	0
Head			+	
Fat body	+++	+		
Spinning gland	++			

does form the pigment. In the prepupa, the enzyme activity of the fat body drops precipitously whereas that of the hypodermis increases. In the pupa, the enzymatic activity of the testis is strongly reduced, and it ceases completely in the adult. In the pupa, on the other hand, the head shows high activity, possibly reflecting the synthesis of pigment in the eye, and in the adult female the highest activity is found in the ovaries, leading to deposition of 3-hydroxykynurenine in the developing egg. This 3-hydroxykynurenine deposited in the ovum can be used by *aa* offspring from heterozygous mothers to form ommochromes, so that the phenotype of the hatching *aa* larvae is determined by the genotype of their mothers, and not by their own genotype. This constitutes a very clear and simple example of so-called maternal inheritance.[11]

The experiments just discussed supply three pieces of information concerning the development of the pattern of ommochrome synthesis. (1) Different organs show different enzymatic activities. (2) The enzymes are synthesized and disappear at specific developmental times. (3) The pattern of enzyme activity bears no relation to the pattern of ommochrome synthesis. In addition to the turning off and on of genes controlling enzyme synthesis we must, then, consider a second cellular character: the ability to react to the presence of kynurenine with pigment formation. This cellular ability to react specifically to a developmental stimulus has been known to embryologists for a long time and has been designated by the term developmental competence. Competence is a crucial concept in embryological considerations.

The time of competence for pigment formation of eyes and testes can be investigated by transplantation of *aa* organs of different ages into wild-type hosts and by injection of kynurenine into *aa* animals at different stages. It turns out that the competence of the testis appears at the time of the last larval molt, or some days later in some of the strains, and disappears at the time of pupation. The eye, on the other hand, maintains its competence through most of the pupal period. The time of onset of competence in the testis is itself under genic control.

THE CHEMICAL AND GENIC BASIS OF COMPETENCE

The nature of competence is, then, another aspect of ommochrome development calling for investigation in chemical terms. Part of the competence of a cell consists in its ability to transform kynurenine into the final pigment, and this ability may be assumed to depend on the presence of those enzymes and conditions necessary for the further reactions of the chain. The adult ovary, for example, has the ability to perform the reaction steps leading up to 3-hydroxykynurenine, but it lacks the conditions to polymerize 3-hydroxykynurenine to ommatin or ommin, which leads to storage of 3-hydroxykynurenine.

Some of the conditions for pigment formation can be investigated by analyzing the structure of the pigment granules. If the pigment is extracted from the pigment granules, e.g., with acidified methanol, colorless granules remain which are stainable with basic dyes[12,13] and can be seen under the phase microscope. They are very small, about 0.2 μ in diameter, and lose their stainability with basic dyes after treatment with ribonuclease. It may therefore be concluded that these so-called core granules contain ribonucleic acids.

In *aa Ephestia* eyes the granules are well developed. Even though they do not contain any ommochrome pigments, they carry the pteridine pigment, in increased amount as compared to the wild type. The testis, on the other hand, does not usually contain colored pteridines, and hence *aa* testes are colorless. It was therefore expected that in the *aa* testis naked core granules would be present. This turned out not to be the case; granules are completely missing in the *aa* testis.[13] If *aa* testes are supplied with kynurenine by, e.g., implantation into a wild-type host, core granules appear after 1 to 2 days and start accumulating pigment. There exists then, in this case, a mutual relationship between the appearance of the core granules and the presence of kynurenine.

The pigment produced from kynurenine is deposited on the core granule, and it is assumed that the core granule itself plays a role in the last steps leading to the formation of the ommochromes. On the other hand, kynurenine itself seems to be required in the *aa* testis for the formation of the core granules. Since the core granules consist of RNA and protein, it must be concluded either that kynurenine induces the formation of a specific component of the core granules in the cell, or that the components are present in the competent cell and need kynurenine as a stimulus to proceed toward their organization into granules.

The formation of core granules is itself under the control of genes. In *Ephestia*, there exists a mutant *wa* (white eyes) which is unable to form ommochrome and pteridine pigments in any of the organs in which they normally occur. Hanser[12] and Maier[14] have shown that *wa* animals lack the core granules; the absence of pigments seems to be a consequence of this lack. The *wa* animals are able to form kynurenine; the formation and distribution of 3-hydroxykynurenine in this mutant have not been investigated. Another white-eyed mutant, *alb*, on the other hand, does contain naked core granules in the eye. In the presence of this gene core granules can be formed in the absence of ommochrome and pteridine pigments.

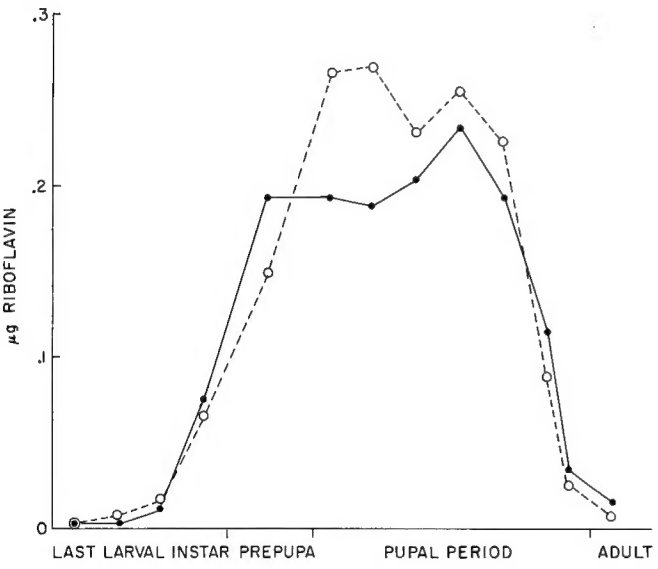

Figure 3. Riboflavin content of the testis in the development of *Ephestia*. Abscissa: time, from last larval molt to adult; ordinate: µg riboflavin per testis; ●———●, strain BII (wild); o– – – –o, strain aa0 (*aa*, ie., red-eyed). BII and aa0 are two strains made isogenic by 32 generations of outcrossing except for the gene *a*. BII reaches a plateau in the prepupa, while aa0 rises higher in the early pupa and starts to drop in the 5-day-old pupa. The secondary peak at 8 days is characteristic of BII and aa0 and does not occur in other strains.

The ability to form the core granules constitutes, therefore, one of the components of the competence to form ommochrome pigments. A second component of competence has been demonstrated: the selective uptake of kynurenine from the blood. Egelhaaf[7] investigated this phenomenon by injecting kynurenine into *aa* larvae. The largest amounts are taken up by the fat body and the intestine and are transformed into 3-hydroxykynurenine. But a certain amount is also taken up by the spinning gland and by the hypodermis, and in the latter it can be transformed into pigment.

The genetic and developmental basis of selective uptake of kynurenine has not been established, but some information is available on the uptake of riboflavin in the testis.[15] *Ephestia* is routinely fed on yellow corn meal enriched with considerable amounts of riboflavin. This riboflavin was found to accumulate in the outer layer of the testis sheath, starting in the middle of the last larval instar (Figure 3), and to stay there until the middle of the pupal period. At the time of its disappearance it appears in the excretory organs; before its appearance in the larval testis, most of the riboflavin is present in the body wall, presumably in the hypodermis. In other words, during the last larval instar riboflavin is released from the hypodermis cells and is taken up selectively by the testis cells. In the pupa, it is in turn released from the testis and stored in the excretory organs.

The mutant gene *wa* inhibits the storage of riboflavin in any of these organs: the substance continues to circulate in the blood. The mutant gene *a*, on the other hand, causes larger amounts of riboflavin to be stored in the testis, its release occurring 1 to 2 days earlier than in the wild type and in a more precipitous way (Figure 3). This is remarkable, since the primary action of *a* seems to be the control of the enzyme tryptophan pyrrolase. Its effect on the level and timing of riboflavin uptake in the testis may therefore be regarded as a secondary effect. Secondary effects of genes have been frequently described in developmental genetics. Conceptually, they do not constitute a great difficulty, since it can be assumed that any specific change in the metabolism of a cell may have secondary consequences for other reactions. But the specific interrelations between primary and sec-

ondary effects of the same gene are in most cases unknown. The ability of particular cells to store or exclude certain substances can be assumed to result in developmental differences describable as differences in competence.

THE TIMING OF PIGMENT FORMATION IN THE EYE

The facts given thus far indicate some of the difficulties of developmental analysis. The formation of the pigments is chemically reasonably well understood, but this does not suffice to answer all developmental questions. The problem of pattern formation has been discussed at three levels: at the level of pigment formation, at the level of enzyme formation, and at the level of competence. At each of these levels a pattern of positive and negative cells is observed. Except for the first level, pigment formation (see Figure 2), the patterns have not been completely analyzed, but they are sufficiently well understood to show that the pattern of pigment formation is largely independent of that of the enzymes directing the individual steps of pigment formation, and may more closely reflect the patterns of competence. Two aspects of competence have been mentioned: the ability to form core granules, and the ability of cells to accumulate substances necessary for pigment formation. It is not intended to limit the concept of competence to these two factors; others may possibly play a role. Furthermore, the dependence of both aspects of competence on genes seems to indicate that competence itself must be the result, direct or indirect, of the synthesis of proteins, probably with enzymatic activity. Such enzymes controlling the uptake of substances by a cell, so-called permeases, are well known from bacterial systems. It may therefore not appear justified to distinguish the level of competence from the level of enzymes. This is, however, convenient for the present analysis, because the term enzymatic level is here restricted to mean those enzymes directly involved in the synthesis of the pigment. Proteins involved in the formation of cellular structures, such as the core granules, and the determination of the properties of cell membranes which may control the ability to take up low molecular substances, may be conveniently distinguished from the pigment-forming enzymes.

The results concerning the pigment pattern of *Ephestia* permit us to consider some of the possibilities for the application of models derived from work with microorganisms to the problem of differentiation. It has been suggested, for example, that the pattern of enzyme synthesis in the cells of an organism may be due to enzyme induction by its substrate. This appears particularly possible for tryptophan pyrrolase, since this enzyme constitutes one of the best investigated cases of enzyme induction in mammals.[16] Egelhaaf,[7] in extensive experiments, was unable to find any effect of injected tryptophan on the level of tryptophan pyrrolase activity in larval and adult *Ephestia*. But Rizki and Rizki[17] found that the same enzyme activity in the fat body of *Drosophila* could be increased by injection of tryptophan. It appears, then, that induction by the substrate may be a possible but not a general mechanism, and that, even for the same enzyme, differences in mechanism may exist in different species. This seems more likely, since variation in the fate of tryptophan in the fat body exists even between strains of *Drosophila*: in most strains, kynurenine is stored as such in the fat body, but a gene, *rc*, enables the fat body to transform kynurenine into an ommochrome. On the other hand, enzyme induction by substances other than the substrate, allosteric effects according to Jacob and Monod,[1] remains an attractive possibility.

Our experience with insect development can also give us some information on the possibility of coordinate repression[18] as a mechanism for the regulation of enzyme synthesis in development. This term refers to a phenomenon observed in microorganisms: in a series of enzymatic reaction steps, accumulation of the last product inhibits the synthesis of enzymes involved in preceding steps. The series of steps leading from tryptophan to the ommochromes constitutes such a series, but the erratic occurrence and distribution of the enzymes in the organs of the *Ephestia* larva and pupa described above contradict the assumption that such a mechanism of regulation is involved in this case.

More positive evidence on the cellular processes involved in ommochrome synthesis has recently been obtained in our laboratory. It concerns the process of pigment formation in the eye during pupal development in the presence of nucleic acid analogues.[19]

The development of pigment in the eye of *Ephestia* pupae proceeds in the following way (Figure 4). Shortly after pupation, a band of red pigment appears in the dorso-caudal region of the eye. This pigment spreads gradually over the posterior part of the eye, where it remains until the end of the

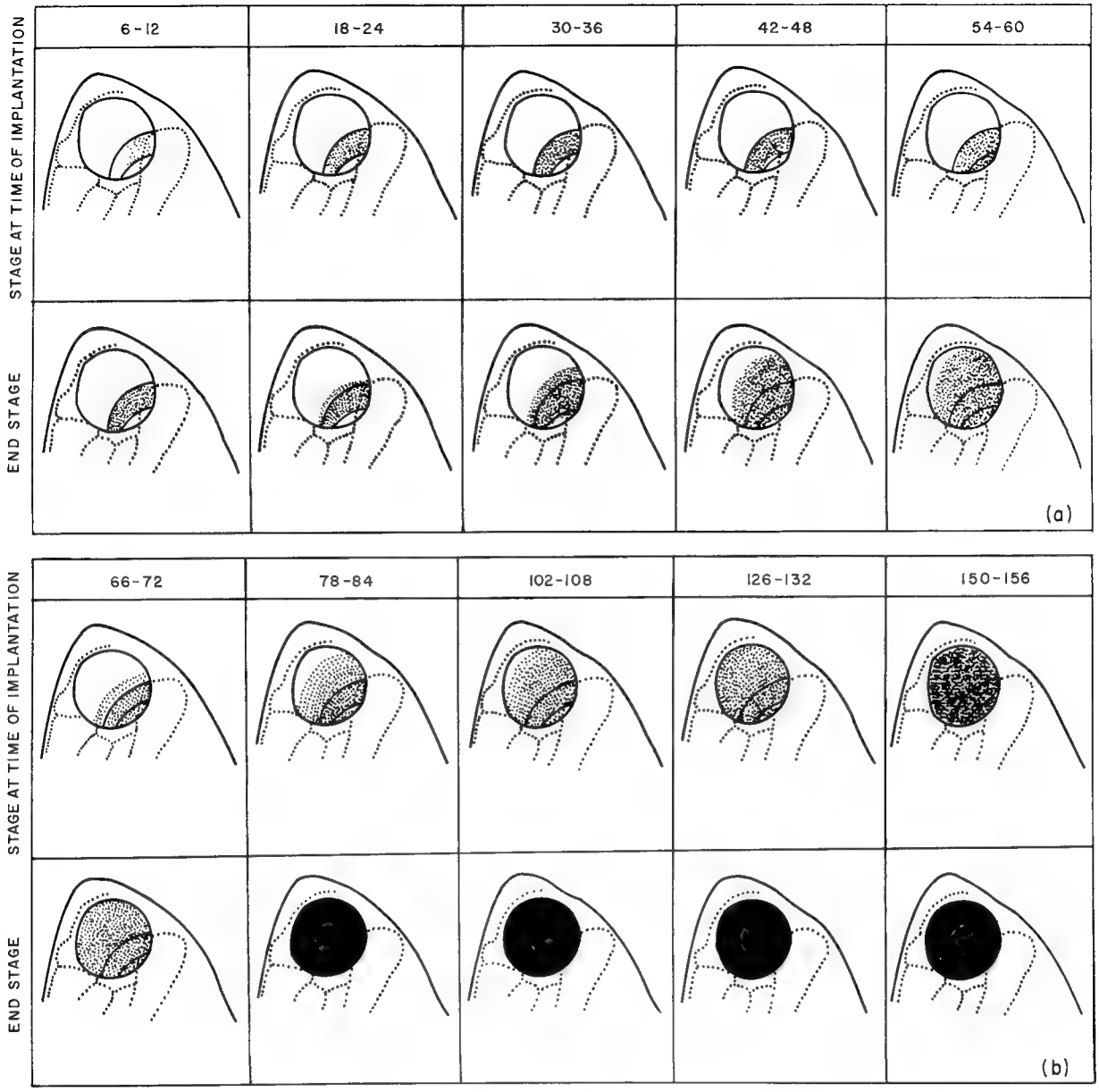

Figure 4. Normal development of the eye pigments in the *Ephestia* pupa, and development under the influence of 5-fluorouracil. (a) Pupae 6 to 60 hr old; (b) pupae 66 to 156 hr old. Upper row, normal development; lower row, final pigmentation reached after implantation of 5-fluorouracil at the time indicated.

third day after pupation. It then advances gradually over the anterior part of the eye, so that it covers the whole surface of the eye on the fifth day. The pigment appears in the form of discrete accumulations, corresponding to the ommatidia, and darkens to a brown color, starting at the dorso-caudal border. On the seventh day after pupation, the whole surface of the eye darkens simultaneously to a homogeneous black color, so that it shows the complete adult pigmentation by the eighth day. In chromatograms, the red ommatin can be demonstrated on the second day after pupation, and xanthommatin on the fourth. It may therefore be assumed that the red-brown pigment spreading over the eye in the first five days of the pupal stage corresponds to the ommatins. Ommins can first be

demonstrated on the seventh day, and the fact that the homogeneous black coloration appears on this day may therefore be due to the synthesis of ommin.

If 5-fluorouracil (5-FU) crystals are implanted into pupae of different ages, development stops, even though the pupae are not killed but survive for at least ten more days, as shown by their oxygen uptake and their ability to move. If a pupa is treated with 5-FU during the first three days after pupation, the spreading pigmentation process does not cover the whole eye, but the amount of pigment in the dorso-caudal part increases to give a dark brown. A pupa treated at this time will therefore show clear separation of the eye into a darkly pigmented part and a completely unpigmented part. The border between the pigmented and unpigmented areas is sharp (Figures 4 and 5); however, the border does not correspond to the area covered by the pigment at the time of implantation, but proceeds farther anteriorly. 5-FU, therefore, does not interfere with the formation of pigment, but it does interfere with the spreading of the pigmented area. Furthermore, the area where the process is stopped extends farther anteriorly than the area actually showing pigment at the time of treatment. In other words, some cells anterior to the pigmented area are already determined to form pigment at the time of treatment; a determination stream precedes the pigmentation stream which passes over the eye. 5-FU inhibits the further advance of the determination stream, but does not affect the differentiation of the cells that are already determined at the time of treatment. It should also be mentioned that the pigment produced after treatment with 5-FU in the first three days consists exclusively of xanthommatin and the red ommatin.

Figure 5. Head of a 12-day-old pupa which had been treated with 5-fluorouracil at the age of 30 to 36 hr.

The result of 5-FU treatment changes drastically on the fourth day after pupation. At this time, 5-FU no longer interferes with the normal pigment formation in the eye; the eyes become completely and homogeneously dark and on chromatographic separation show normal amounts of ommatin and ommin. It should be noted that ommin appears in 5-FU treated pupae, as in normal animals, on the seventh day; in other words, the cells become determined to form ommin on the fourth day, even though the pigment does not actually appear until the seventh day.

These data permit us to resolve the pigmentation process of the eye into two components: the formation of the ommatins and the formation of ommin. This conclusion is in agreement with the fact that the gene *br* inhibits the second process without interfering with the first. More important is the fact that the experiments with 5-FU lead us to introduce the term determination and to distinguish it from differentiation. At certain times of development, a cell becomes determined to form ommatins or ommin, and after this time 5-FU can no longer interfere with the formation of the pigment. The concept of determination has been much used in classical embryology and is based on the finding that before differentiation there occurs a critical period after which the cells pursue a developmental path independently of experimental influences. Even though this concept is somewhat indefinite from an operational point of view, since it is impossible to confirm or reject the hypothesis that the determined cells will continue differentiation under all conceivable conditions, it is meaningful in a particular set of experiments. It means that the cells that are going to carry out pigment synthesis in the presence of 5-FU must in some ways be different from those that are not able to do so. The experiment also shows that the processes of determination for the formation of ommatins and ommins proceed in different ways: in the former case a determination stream passes over the eye, while in the case of the ommins all cells of the eye are determined simultaneously.

These experiments do not yet permit us to define the concept of determination in chemical terms.

5-FU is incorporated into RNA, and it is therefore possible that one of the components of the information transfer from the genes to the proteins is affected by this treatment. The involvement of RNA in processes of determination has been postulated by embryologists for a long time. But 5-FU also has an inhibitory effect on DNA synthesis. The latter action seems, however, less likely to form the basis of the 5-FU effect on eye pigmentation, since 5-fluorodeoxyuridine, which would be expected to affect DNA synthesis in the same way as 5-FU, is without effect on pigment formation. It appears more likely that in the experiments 5-FU affects the synthesis of an RNA component, possibly the messenger RNA which is the primary product of the DNA carrying the genetic information. It has been established in microorganisms that 5-FU is incorporated into newly formed RNA in the place of uracil, which results in the formation of an abnormal RNA which cannot carry out its function. If it is assumed, as was postulated in the Introduction, that differentiation consists in the activation of particular genes in particular cells, and since it is generally assumed that this activation shows itself by the production of messenger RNA and initiation of its activity in synthesizing specific proteins, the period of sensitivity to 5-FU should coincide with the period preceding formation of the messenger RNA for a particular enzyme, so that in the presence of 5-FU a "fraudulent" messenger RNA would be formed. After this "critical" period, 5-FU would not have this effect on messenger RNA, which probably indicates that a sufficient amount for the completion of the process has been produced. Since, in the case of the ommin pigment, almost three days elapse between the critical period and the formation of the pigment, it must be concluded either that this messenger RNA has a considerable lifetime, or that the processes set in motion by the production of this substance need three days for their completion.

PIGMENT FORMATION AS PART OF THE MORPHOGENESIS OF THE EYE

Up to this point, the formation of ommochrome pigments has been treated as a more or less autonomous chemical process going on in certain cells at certain times. The 5-FU experiments show, however, that pigment formation is dependent on processes occurring in neighboring cells: the determination stream preparing the cells for ommatin formation starts from a determination center in the dorso-caudal region of the eye and spreads gradually, in the course of three days, over the surface of the eye. A more thorough investigation of the 5-FU effect on the development of the *Ephestia* eye, carried out by R.B. Imberski,[20] demonstrated that pigment formation is a partial process of the differentiation of the eye and must be considered in connection with developmental processes occurring in other parts of the eye.

In order to analyze these processes, the normal development of the *Ephestia* eye will be briefly described. Figure 6 is a photograph of the completely differentiated eye of a late (246 to 252-hr-old) pupa. The eye like all insect eyes consists of a large number of visual elements, the so-called ommatidia. The structure of two ommatidial elements is schematically represented in Figure 7. The actual visual elements, the retinulae, consist of eight long, extended cells which have their nuclei and pigment in their distal parts, and a rodlike structure, the rhabdom, in their proximal parts. One basal retinula cell, its nucleus covered by a cap of pigment granules, is found close to the basement membrane. The distal part of the ommatidium is mainly composed of the crystalline cone, a transparent structure stained rather darkly in Figure 6. It is surrounded by the primary pigment cells, rather narrow, inconspicuous structures in *Ephestia*, and the distally located four crystalline cone cells. The most distal structure, the cornea, is a modifi-

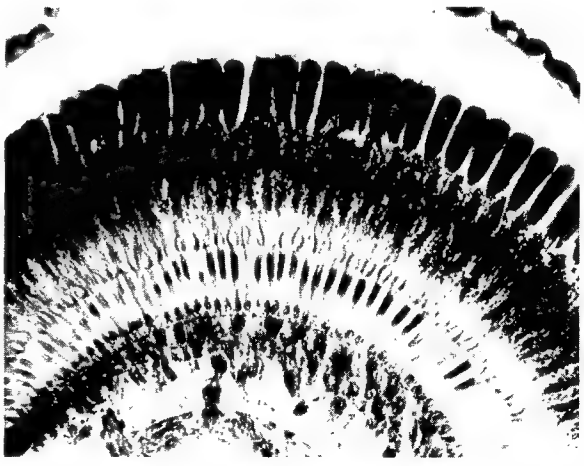

Figure 6. Part of the eye of an old (246 to 252 hr) pupa. The cornea has been lifted off, but can be seen in the upper right and left corners. The crystalline cones stain darkly. (Courtesy Dr. R.B. Imberski.)

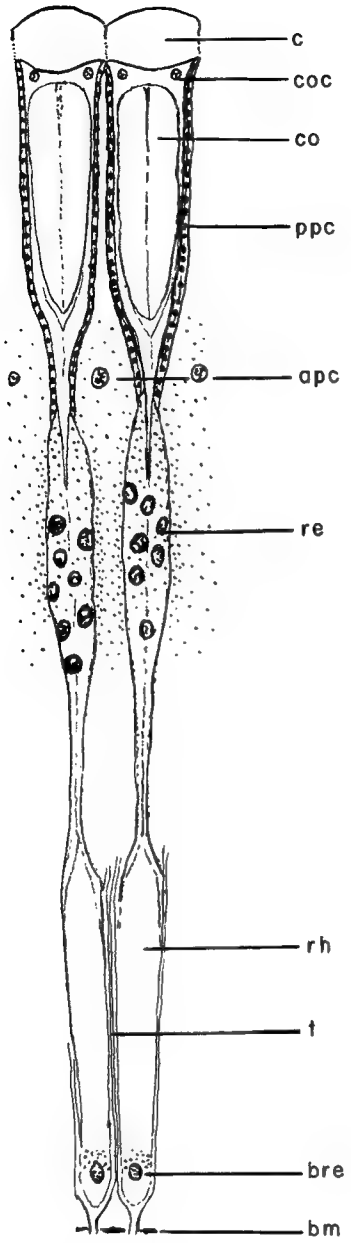

Figure 7. Schematic drawing of two ommatidia from an adult eye. *c*, cornea; *coc*, crystalline cone cells; *co*, crystalline cone; *ppc*, primary pigment cells; *apc*, accessory pigment cells; *re*, retinula cells; *rh*, rhabdom; *bre*, basal retinula cell; *bm*, basement membrane; *t*, trachea. Stippling indicates the location of pigment.

cation of the cuticle, which has become separated from the eye proper in Figure 6 but can be seen in the upper right and left corners. The space between the ommatidia is filled by the heavily pigmented accessory pigment cells.

Immediately after pupation, the whole eye is a high epithelium containing several layers of nuclei. The cell boundaries are not distinct. At the end of the second day, the whole epithelium stretches and thus becomes thinner, and the rudiments of a structure corresponding to the ommatidia, i.e., retinula cells and crystalline cone cells, become visible (Figure 8.) It should be mentioned that assigning prospective fates to individual cells at this stage is hypothetical; it seems valid, however, to regard the central cells of the structure as retinula cells and crystalline cone cells. In the following three days, the organization of the presumptive ommatidium is in principle completed; the four distal cells of each ommatidial complex secrete vacuoles which fuse and become organized into the crystalline cone; the proximal cells form clumps of pigment and differentiate into the retinula cells. In later development, the ommatidia stretch strongly in a proximo-distal direction, the cornea differentiates in the distal part, and the

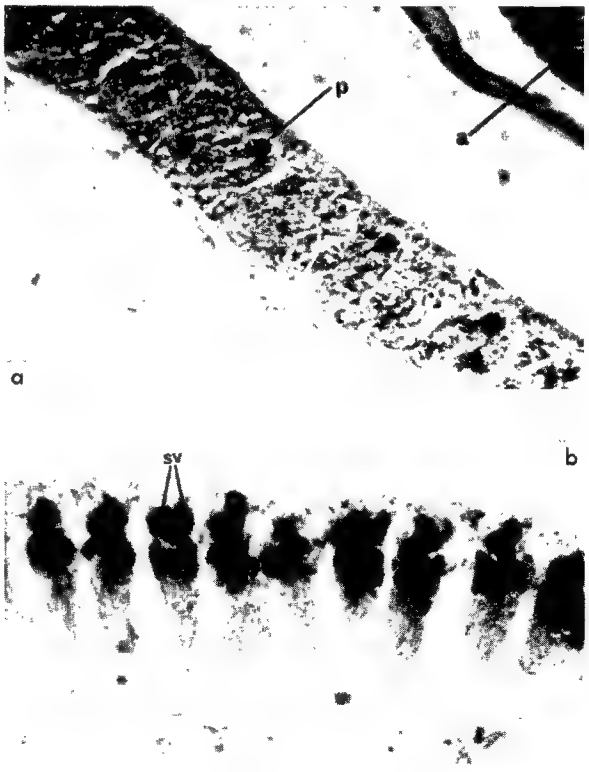

Figure 8. (*a*) Eye epithelium of *Ephestia* pupa at 42 to 48 hr; *p*, pigment; *a*, antenna, which lies close to the upper corner of the eye. (*b*) Part of the eye of a 12-day-old pupa which had been treated with 5-fluorouracil at a stage corresponding to that in (*a*), i.e., 42 to 48 hr. (Courtesy Dr. R.B. Imberski.)

rhabdom in the retinula cells. The accessory pigment cells become distinctly visible on the fifth day, develop their pigment on the seventh day, and elongate to fill out the area between the ommatidia on the eighth day.

All the early differentiations in the eye appear in the form of spreading processes starting from the same determination center in the dorso-caudal region of the eye. The earliest visible processes starting in this region are the formation of pigment clumps and the appearance of nerve fibers connecting the visual elements with the brain. Similarly, the formation of the ommatidial complexes, shown in Figure 8a, starts on the second day in the dorso-caudal region of the eye and spreads over the whole eye by the fifth day after pupation. Finally, the formation of vacuoles in the crystalline cone cells, then their coalescence, and their organization to form the crystalline cones depend on spreading processes which start at the same center on the third, fifth, and sixth day, respectively, and reach the anterior border of the eye at about the same time, on the sixth day. The later developmental processes – the development of the accessory pigment cells, the formation of the rhabdom, and the differentiation of the cornea – occur simultaneously all over the eye.

All the developmental processes can be inhibited by treatment with 5-FU at appropriate times. An example is given in Figure 8. In Figure 8a, the state of the dorsal part of the eye at 42 to 48 hr is shown. The epithelium is differentiated into oval ommatidial complexes, each carrying a clump of pigment, but otherwise no structures are visible. If 5-FU is introduced at this time, the ommatidia develop to the state shown in Figure 8b: they are well differentiated from each other, the amount of pigment has increased, and in the distal cells vacuoles have been formed which have fused but have not formed clearly organized crystalline cones.

It becomes clear that the first pigmentation process defined by 5-FU treatment, the spreading process, concerns pigment formation in the retinula cells, while the second corresponds to pigment formation in the accessory cells. The latter has a sharp critical period for treatment with 5-FU. Treatment up to 72 hr inhibits the differentiation of the accessory pigment cells, including their pigmentation, while treatment after this time has no influence on their development: they assume their normal shape and pigmentation. Similar sharp, sensitive periods are found for the formation of the cornea (60 hr) and of the rhabdom (144 hr). For the earlier processes, on the other hand, 5-FU stops the determination stream, just as it stops the pigment determination stream affecting the retinula cell pigment. For instance, application of 5-FU at 42 to 48 hr permits the organization of ommatidial complexes and the formation and fusion of vacuoles in the crystalline cone cells in the dorso-caudal part of the eye (see Figure 8b), but not in the anterior part; treatment at 66 to 72 hr, however, does not interfere with the formation of these structures all over the eye.

The main conclusion to be drawn from these experiments is that at the earliest stages considered, at a time when the eye appears as a simple high epithelium, there already must be a certain amount of differentiation of the eye, what Stern[21] has called a prepattern. The determination center must in some way be distinct from the other parts of the eye; furthermore, the future retinula cells must already be distinct in some ways from the remaining cells, since they react to the determination stream with pigment formation and outgrowth of nerve fibers, while the other cells do not. At later stages different cells react with different activities on the stimulus proceeding from the determination center. It is not clear whether a series of different determination streams originates at the same center, or whether different cells react differently on the same determination stream at different times or at different concentrations. At all events, pigment formation turns out to be, in every instance, one aspect of the determination of cells. This is most clearly seen in the accessory pigment cells in which 5-FU treatment before the critical period inhibits all differentiation processes which in normal development occur in a series extending over several days.

DISCUSSION AND CONCLUSIONS

In an analysis of the development of the ommochrome pattern of insects we are dealing with a process that is chemically understood to a reasonable degree. The process has been resolved into a number of subprocesses: (1) In ommochrome synthesis, different cells differ in the enzyme patterns involved. This is usually ascribed to the activation of different genes at different times of development. (2) The actual synthesis of the pigment is preceded by a process of determination which must effect a change in the cells without visible representation.

This may not be different in principle from the activation of particular genes, which results in the synthesis of particular enzymes. It means only that differentiation is not a suddenly occurring event, but consists in the initiation of a process continuing for some time. (3) The concept of competence again may not in principle be different from the others, except that it involves interaction between different types of cells. Only when a substance like kynurenine is released by cells different from those producing the pigment does the concept of competence have an operational meaning. In such a case, competence includes selective uptake of the substance in question and the chemical and morphological conditions necessary for the formation of the final product. It can be seen that all these concepts do not necessarily indicate fundamentally different processes. The acquisition of competence may very well, in another experimental setting, be described as determination. It is in this sense that the models of Jacob and Monod,[1] describing a number of possible repressing and activating feedback systems at the genic level, give us an understanding of developmental processes in very general terms.

But the student of embryology is not satisfied by an interpretation in such general terms. He wants to know more about the system of interactions not only within the cell, but also between the cells of the developing organism. It appears from our analysis of the pigmentary system of insects that such an interpretation in general terms may well be impossible. Ommochromes occur as eye pigments in all insects, and probably in all arthropods, and have in addition a species-specific pattern of distribution in the arthropod body. The chemical basis of pigment synthesis is relatively simple, consisting of a limited number of enzymatically controlled steps. Each of these steps is under the control of a genetic locus, and these loci seem to be independently activated. The pigment is formed in those cells in which all the conditions for the formation of pigment granules are present, i.e., kynurenine, the enzymes transforming kynurenine to ommochromes, and the core granules or the conditions necessary for the organization of these cellular structures in the presence of kynurenine. Every one of these processes can in principle be described in molecular terms, although not all have yet been defined in this way. The coordination of the activities of the genes involved, which results in the pattern of gene expression, may be assumed to be the result of the evolutionary history of the organism. This accounts for the variability of the partial processes of pigment formation and of the pattern of pigment distribution, not only between *Ephestia* and *Drosophila* but also between *Drosophila* species and between different Lepidoptera. It is difficult to think at the same time in terms of mechanism and of history. But it appears to me that it is exactly in this area that the conceptual difficulty in the analysis of development is situated. The fundamental mechanisms may well turn out to be simple and to involve nothing more than the control of genic activities. The organization of the different genic activities and partial processes into a coordinated pattern may be unique for every species and defy a generalized picture applicable to all organisms. It is this part of development that can probably be understood only from the standpoint of evolutionary considerations.

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